Characterization of Two Steroidal Olefins in Nonfat Dry Milk

ABSTRACT

Two steroidal olefins were isolated from the hydrocarbon fraction of a n-hexane extract of nonfat dry milk. They were characterized as 24-methyl-Δ^2-cholestene (Δ^2-campestene) and 24-ethyl-Δ^2-cholestene (Δ^2-sitostene) by gas chromatography, mass spectrometry, and synthesis.

INTRODUCTION

Most of the investigations on steroids present in milk have been conducted with anhydrous milk fat (AMF). Cholesterol is by far the major steroid compound in milk. Brewington, et al., (1) identified small amounts of dihydrolanosterol and β-sitosterol and confirmed the presence of lanosterol, first proposed by Morice (2). Campesterol also has been found in milk fat (C.R. Brewington, personal communication). Eisner, et al., (3) reported the presence of the pentacyclic triterpenoid β-amyrin. The only nonsterol identified in milk fat is Δ^7-cholesten-3-one reported by Parks, et al., (4). Little work has been done on steroids present in the lipid phase extracted from skim milk, even though this is known to contain higher concentrations of cholesterol, carotenoids, and vitamin A than the fat extracted from normal milk (5). Accordingly, it seemed reasonable to expect that the lipid phase extracted from nonfat dry milk (NFDM) might contain constituents either not present in milk fat or present in such low concentration as to have escaped detection. With this idea in mind, the present work was undertaken with the purpose of identifying novel steroid compounds in NFDM.

EXPERIMENTAL PROCEDURES

Gas Liquid Chromatography (GLC) and Mass Spectrometry (MS)

The GLC analysis was performed using a Hewlett-Packard 5750B gas chromatograph equipped with a flame ionization detector. The column was 2.4 m in length x 3.2 mm outside diameter, stainless steel, treated with dimethylchlorosilane (DMCS), and packed with 3% JXR on 80-100 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The effluent was split 10:1 for trapping purposes. The temperature was programed from 90-220 C at a rate of 6 C/min. The carrier gas (He) was supplied at a head pressure of 2.8 kg/cm^2. The GLC-MS system was an LKB-9000, operating at an ionizing energy of 70 eV, source temperature 290 C, accelerating voltage 3.5 kv, separator temperature 240 C; the chromatographic column and conditions were as just described. The high resolution mass spectra were determined with a Dupont CEC 21-110B double focusing mass spectrometer at 70 eV. The sample was introduced via a direct probe at a source temperature of 115 C. The spectra were recorded on photoplates, and accuracte mass measurements were made by means of a comparator, with perfluorokerosene as internal standard.

Synthetic Procedures

All melting points (mp) are uncorrected. 3-Cholestanyl acetate, mp 107-108.5 C, was prepared according to Benveniste, et al. (6). 3-Cholestanyl p-toluenesulfonate, mp 135-136 C, was prepared according to Douglas, et al. (7).

Δ^2-Campestene and Δ^2-sitostene were synthesized starting from a commercial sample of β-sitosterol (Chemical Procurement Laboratories, College Point, N.Y.). This particular sample melted at 136-137 C after 2 crystallizations from ethyl acetate and 1 from ethanol. GLC and MS of the recrystallized product showed that it was a mixture of β-sitosterol (85%) and campesterol (15%). Hydrogenation of this mixture, according to Hershberg, et al., (8) yielded a solid (mp 134-135 C) which gave a negative bromine test (9) after crystallization from ethanol. The assumption of two hydrogen atoms was confirmed by MS. The remaining steps in the preparation of the Δ^2-olefins were those used by Fieser and Dominguez (10) for the preparation of Δ^2-cholestene. The crude olefin mixture melted at 69 C. Thin layer chromatography (TLC) on silicic acid impregnated with $AgNO_3$ and developed with n-hexane (J.T. Baker Chemical Co., Phillipsburg, N.J.) indicated that the crude product contained ca. 10% nonolefinic impurity (R_f = O). Purification over acid alumina (J.T. Baker Chemical Co.), with n-hexane as eluent, yielded a crystalline solid (mp 75-76 C) consisting of a 1:9 (GLC) mix-

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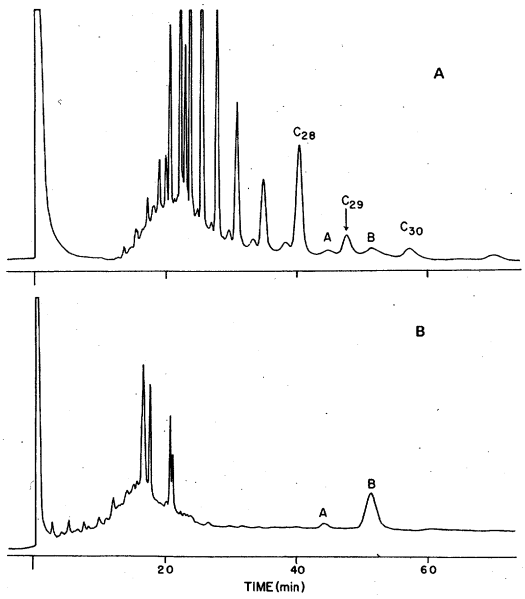

FIG. 1. Flame ionization detector chromatogram of an n-hexane extract of nonfat dry milk: (A) after column chromatography over hydrated alumina (total eluate) and (B) after further purification over acid alumina (Fraction NFDM, AB,3 and 4).

ture of Δ^2-campestene and Δ^2-sitostene (TLC and MS).

Isolation of the Steroidal Olefins from Nonfat Dry Milk

NFDM (3.5 kg), purchased at a local supermarket, was hydrated to a 9% water content, and extracted in a Soxhlet extractor with n-hexane in 350 g batches. The hexane extract was dried over Na_2SO_4 and evaporated to dryness on a steam bath under a stream of nitrogen. The waxy residue was taken up in 2 ml n-hexane and placed on a 50 g column of acid alumina previously hydrated to a 6% water content. The column then was eluted with 200 ml n-hexane and the eluate collected and reduced to dryness as above. The residue was dissolved in 500 μliter n-hexane and placed on a 13.5 cm x 0.7 cm column of acid alumina which then was eluted with n-hexane. Four 5 ml fractions were collected (NFDM, A-D). The first two fractions were combined, taken to dryness, and rechromatographed over acid alumina as above. Again four 5 ml fractions were collected and identified as NFDM, AB,1-4. Each fraction was reduced to 50 μliter and subjected to GLC-MS analysis. More NFDM (2.8 kg) was extracted exactly as above, and the appropriate fractions (NFDM, AB,3 and 4) were used to obtain samples for the high resolution mass spectrometric analysis.

RESULTS AND DISCUSSION

Figure 1A shows the GLC of the n-hexane extract of NFDM after column chromatography over hydrated alumina. This chromatogram is similar to that obtained with the hydrocarbon fraction of AMF (11). Continuous scanning with the GLC-MS system confirmed a hydrocarbon composition with traces of polychlorinated biphenyls. However, the quantitative distribution of the various components is not identical with that observed in AMF (11).

Two mass spectra were recorded for two unknowns, hereafter referred to as A and B, associated with the peaks marked by these same letters (Fig. 1A). These unknowns were investigated further. Purification, accomplished by two passes through columns of acid alumina, afforded a marked enrichment of A and B, which were found in fractions NFDM, AB,3 and 4 (Fig. 1B). The low resolution mass spectra of A (molecular weight 384) and B (molecular weight 398) are shown in Figures 2 and 3, respectively. Both compounds were isolated in a reasonably pure state by trapping off the JXR column and were subjected to high resolution mass spectra, which afforded exact mass measurements and elemental compositions. These are 384.3750 ($C_{28}H_{48}$) for A, and 398.3897 ($C_{29}H_{50}$) for B. The high relative intensity of the molecular ions in the mass spectra of the unknowns (Figs. 2 and 3) and the late elution of the compounds from the JXR column, relative to the C_{28} and C_{29} n-alkane counterparts, are indicative of cyclic or polycyclic molecules. The overall appearance of both spectra (primarily the fragments of m/e 257, m/e 203, m/e 215, m/e 216, and m/e 217, and the presence of M-15 fragments) strongly suggest steroidal structures (12-14). Because the molecules of the unknowns contain each five sites of unsaturation, (if we assume a four-ring steroidal system), these molecules also must contain either a double bond or a fifth cycle. The relatively low intensity of the peaks of m/e 257 excludes the presence of a double bond in the side chain. In fact, in a detailed study, Wyllie and Djerassi (14) established that mass spectra of steroidal olefins with unsaturation in the side chain have a rearrangement ion at m/e 257, which is usually much more intense than the molecular ion and sometimes is the base peak. A prominent feature of the spectrum of A is a strong m/e 330 peak (elemental composition $C_{24}H_{42}$). This corresponds to the loss of a neutral C_4H_6 fragment from the molecular ion. The elimination of butadiene is highly characteristic of Δ^2-steroids (13,15) and results from a retro-Diels-Alder decomposition of ring A following electron impact. The analogous frag-

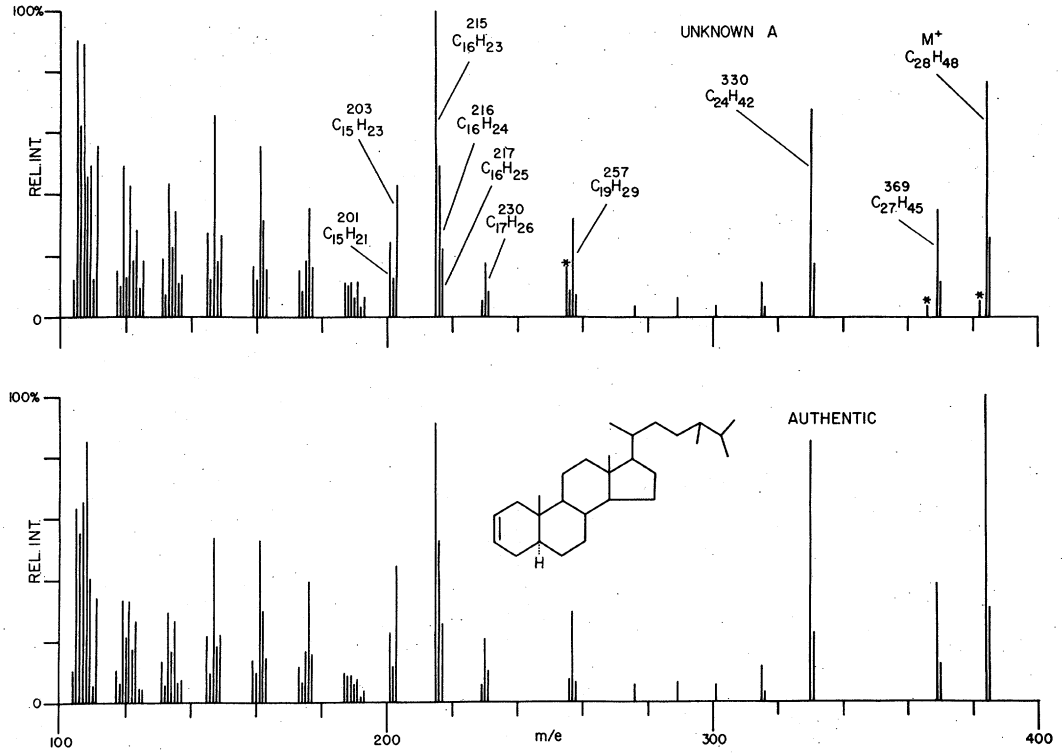

FIG. 2. Mass spectra of compound A and of synthetic Δ^2-campestene. * = peaks from impurity (see "Results and Discussion" section).

ment in the spectrum of compound B occurs at m/e 344 (elemental composition $C_{25}H_{44}$). The fragment of m/e 257, then, must be due to the loss of the side chain at C-17. On the basis of the above evidence, the compounds A and B appear to be Δ^2-steroidal olefins of the ergostane and sitostane series, respectively. In Figure 2, the mass spectrum of A is compared with that of the synthetic Δ^2-campestene. In Figure 3, the spectrum of B is compared with that of synthetic Δ^2-sitostene. The peaks marked with asterisks are due to impurities, possibly steoridal diolefins which are eluted along with A and B. The mol wt of such impurities are 382 and 396 and both have their base peaks at m/e 255. The association of the peaks of m/e 382 and m/e 396 with that of m/e 255 was confirmed by their behavior in multiple fast scanning. The retention times (JXR column) of compounds A and B are identical with those of synthetic Δ^2-campestene and Δ^2-sitostene, respectively. On the basis of the excellent agreement between the mass spectra and chromatographic behavior of the unknowns and those of the synthetic olefins, A is identified as a 24-methyl-Δ^2-cholestene and B as a 24-ethyl-Δ^2-cholestene. Because A and B occur naturally, the location of the methyl and ethyl groups (respectively) is assumed to be at C-24. The stereochemical configuration at C-24 in both compounds remains undetermined.

We estimate that the concentration of A and B in the samples of NFDM used in this study is less than 1 ppm. Their origin, or their precursors, can be objects for speculation. The hypothesis that A and B might be artifacts arising from the corresponding stanol esters, by elimination of a molecule of acid during column chromatography, must be ruled out, because 3-cholestanyl acetate, and even 3-cholestanyl p-toluenesulfonate, did not undergo such elimination by chromatography over acid alumina under the conditions described above. Δ^2-Steroidal olefins have been prepared from tosylate esters with alumina but only after prolonged treatment (7). Thermal or electron impact induced elimination of one molecule of acid from hypothetical stanol ester precursors of the unknowns during MS (16) also must be ruled out, because the column chromatographic procedure employed for the purification of the extract of NFDM completely removes compounds having oxygenated functions.

Phytene, a nonsteroid constituent of the

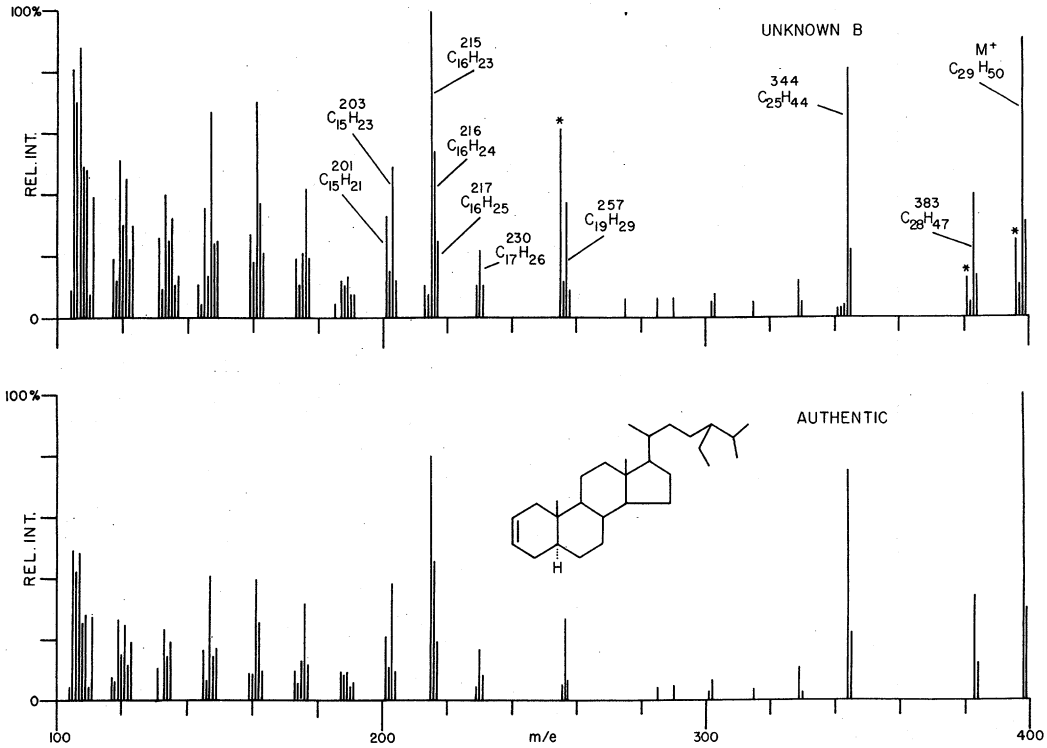

FIG. 3. Mass spectra of compound B and of synthetic Δ^2-sitostene. * = peaks from impurity (see "Results and Discussion" section).

unsaponifiable fraction of AMF (11), was not detected in the present investigation. Conversely, when AMF (prepared from mixed-herd milk, Beltsville, Maryland) was extracted as previously described (11) and the extract worked up in the same manner as the extract of NFDM, no trace of steroid olefin could be detected by GLC-MS. Phytene probably is introduced into milk via feed. The steroidal olefins A and B or their precursors also are almost certainly of plant origin. If this is the case, their occurrence in the lipid fraction of milk would depend upon the nature of the feed. As mentioned in the introductory section, certain lipoid constituents tend to concentrate in the skim milk portion. The lack of uniformity in the occurrence of phytene and of the two steroidal olefins might be another example of preferential distribution or association exhibited by certain constituents. At any rate, given the suspected plant origin of phytene and of A and B and the uncertainty about their precursors, these compounds may not be regarded as normal constituents of milk.

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